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Quantitative Lethality of Ozone for Escherichia Coli

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IRVING DAVIS, Major, USAF (MSC)

Astromicrobiology Section
Microbiology–Cellular Biology Branch

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A series of studies designed to provide an understanding of the biologic activity of ozone at the cellular level is described. A critical analysis of the quantitative lethality of ozone was undertaken.

Maximum lethal effects of ozone over a wide concentration range (0.1 to 1.5 μg. per milliliter) were expressed in 1 minute or less on the bacterial population (approximately 2×10^9 cells per milliliter). Lethality in this range of ozone was from 63 to 99.5 percent. The number of cells surviving exposure is a function of approximately the cube of the initial cell concentration. This holds true over a wide range of ozone values (0.1 to 1.74 μg. per milliliter). Percent kill increased sharply over a narrow range of ozone concentrations (0 to 0.4 μg. per milliliter) and then leveled off. The ozone tolerance of strains B and B/r of *Escherichia coli* appears to be similar, if not identical.

Several factors which contribute to an understanding of the biologic activity of ozone are presented. The importance of free radicals in biologic processes is discussed. Literature on the formation of these radicals during the absorption of ozone in aqueous solution is reviewed.

Atmospheric ozone is formed by the photodissociation of the oxygen molecule by ultraviolet sunlight of short wavelengths. This same source of energy at longer wavelengths also dissociates the ozone molecule so that photochemical equilibrium is established in the atmosphere (1). In the laboratory ozone may be produced by similar ultraviolet activity or by ozonator discharge in oxygen or air.

Among the atmospheric gases, ozone is chemically the most active next to atomic oxygen (2). Laboratory studies with ozone have shown that this molecule reacts with amino acids and proteins (3), with nucleic acids and their derivatives (4), and with other compounds (5), all of which are biologically essential.

Reports in the literature on the effect of ozone on microorganisms are scant. Studies relating ozone to food preservation by the suppression of molds and bacteria (6, 7), to aerial disinfecting (8), to purification of drinking water (9), and to virucidal (10), amebicidal (11, 12), and other effects (6, 13, 14, 15) have been reported. A critical analysis of the lethality of both pure and atmospheric ozone for microbial populations is lacking.

A series of studies designed to provide an understanding of the biologic activity of ozone at the cellular level is described. The quantitativeness of ozone lethality for bacteria was investigated. Evidence for the critical functions of certain factors operative during the killing process is presented. The mutagenic activity of ozone is discussed in a subsequent paper (16).

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MATERIALS AND METHODS

Ozonators

The use of a commercial laboratory ozonator, the Welsbach model T-23, was obtained

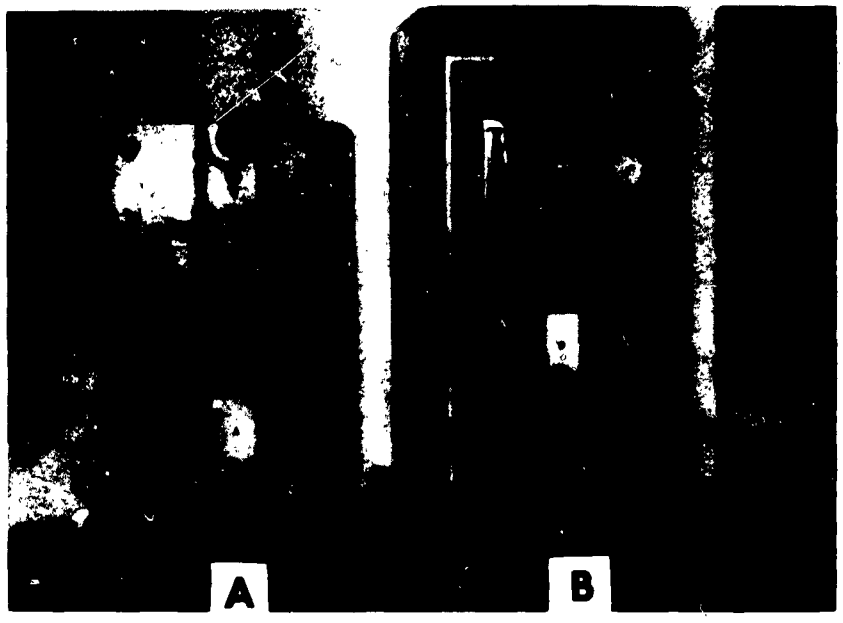

FIGURE 1

Ozone generators (ozonators). **A.** *Fabricated instrument;* **B.** *Commercial, Welsbach model T-23 instrument.*

(fig. 1). This instrument operates on the principle of silent electric corona discharge. It contains an internal high-voltage transformer, voltage regulator, voltmeter, circuit breaker, air or oxygen pressure-maintaining valve, sampling valve, throttling valve, pressure gage, pressure relief valve, and flowmeter. All lines carrying ozone output from the ozonator are glass, except for small pieces of Tygon tubing connections where necessary. Glass-to-glass connections are made with ground-glass ball-and-socket joints.

Gas absorption bottles

Standard gas absorption bottles were procured (Corning catalog No. 31760). These are of 250-ml. capacity and contain coarse porosity fritted glass filters. All bottles were modified by annealing a ground-glass ball joint to the influent arm and a similar socket joint to the effluent arm. The internal tube was modified further after experimentation with several types of glass filters and after advice had been received from several sources (17, 18). The tube finally incorporated into all gas absorption bottles was the Allihn type of absorber and will be referred to as the bell-bubbler. A complete description for its fabrication has been published (17). Several types of gas absorber tubes are shown in figure 2.

Preparation of ozone in aqueous solutions

Ozone was quantitatively produced by the water-cooled corona discharge laboratory ozonator (fig. 3). Dry, clean oxygen, reduced to 15 p.s.i. from the tank, was admitted into the ozonator where the pressure was maintained at 8 p.s.i. Primary voltage was operated between 70 to 115 volts, depending upon ozone concentration desired. The ozone-oxygen flow was passed out of the ozonator at a rotameter setting of 0.1 cubic foot per minute (air at 70° C. and 8 p.s.i.g.). Successively, this O_3-O_2 flow passed through a bell-bubbler absorption bottle containing 200 ml. of distilled water, then through a soda lime chemical adsorber, and finally, through a wet test meter to determine total gas volume. These ozonated-water solutions in the absorption bottles constituted stock

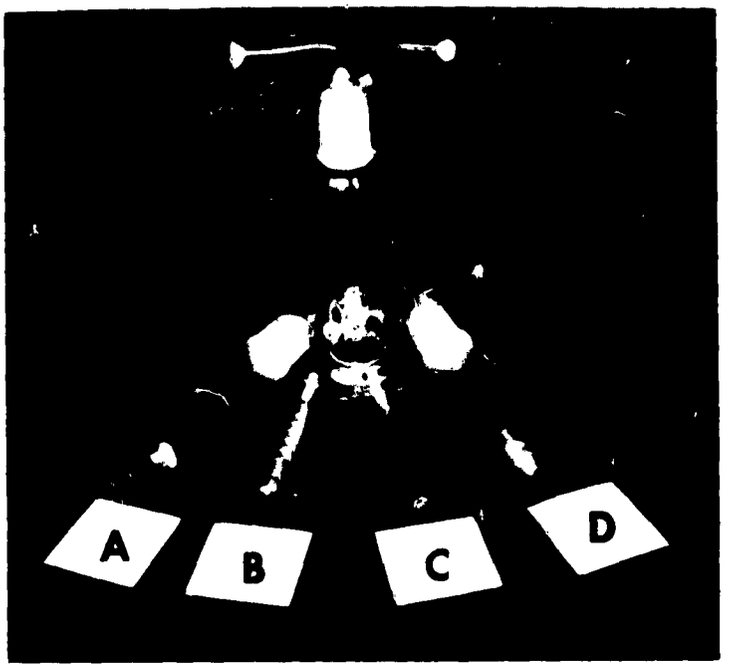

FIGURE 2

Gas absorption bottle and assorted tubes. **A.** *Fritted glass, coarse porosity, tube;* **B.** *Spiral tube;* **C.** *Sparger tube;* **D.** *Bell-bubbler tube.*

solutions for the biologic studies. All operations were carried out at room temperatures (24 to 27° C.).

Analysis of ozone in aqueous solution

The analytic procedure adopted for determining the concentration of ozone in aqueous solutions was a modification (19) of the spectrophotometric method of quantitating ozone in air published by Smith and Diamond (20, 21). Twenty-ml. aliquots of the ozonated-water stock solution were volumetrically pipetted or automatically delivered into 30 x 200 mm. tubes containing 5 ml. of 10 percent alkaline potassium iodide (in 0.1 N NaOH). The tubes were shaken and 1.5 ml. of approximately 3 N acetic acid (1 part glacial acetic acid plus 5 parts of distilled water) were introduced. Optical density of the solution in a calibrated cuvet of 1 cm. light path was read at 352 mμ in the Beckman DU spectrophotometer at intervals of 4 minutes and 20 minutes after acidification. The reference cell contained distilled water. A chemical blank was carried through the same procedure and was reflected in the final net optical density. All water used was distilled (commercial-injectable type). Two chemical determinations were carried out to bracket the biologic tubes. Duplicate determinations which differed by more than 10 percent of each other were discarded. Net average optical densities obtained were converted to milligrams O_3 per liter of water from a curve previously established with standard potassium iodate (fig. 4) (19). These ozone concentrations were corrected for the biologic systems by applying the appropriate dilution correction factor. Ozone values are stated as micrograms per milliliter of the biologic system.

Cultures

The microorganisms selected for study were strains of *Escherichia coli,* because of their extensive employment in radiation and genetic

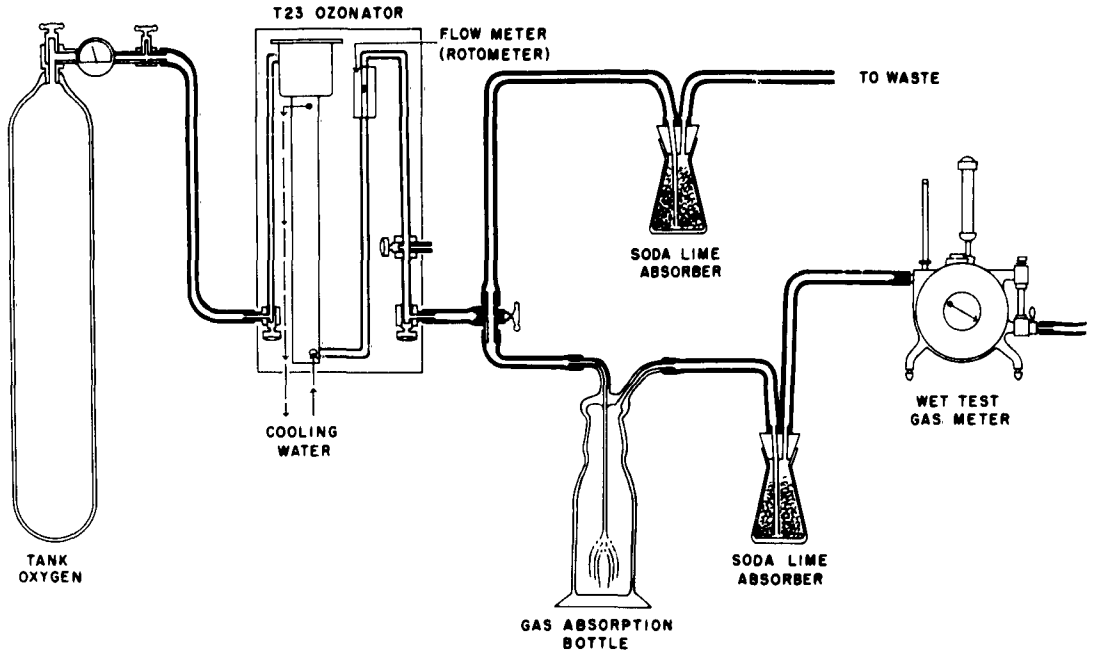

FIGURE 3

Schema of experimental apparatus for the preparation of ozone in aqueous solutions by use of commercial Welsbach model T-23 ozonator.

analyses. *E. coli* B, ultraviolet-sensitive, and B/r, ultraviolet-resistant, were used during the ozone lethality studies. Nutrient stock slants were prepared from the cultures originally received after checks for purity were completed. Daily cultures were prepared by suspending the cells of one stock slant into 100 ml. of nutrient broth contained in a shallow layer in a 500-ml. Erlenmeyer flask. These nutrient broth cultures were incubated at 37° C. for 18 hours without shaking. All nutrient broth and agar cultures contained 0.5 percent NaCl. After incubation, these cultures were centrifuged at 10,000 r.p.m. for 10 minutes, followed by two successive washes and centrifugations in M/15 phosphate buffer (pH 7.32). The cells were then diluted in M/15 phosphate buffer to the desired optical density in the DU spectrophotometer. Viability curves had been previously established relating optical density to total number of viable cells under similar conditions of culture preparation. One ml. of this standardized culture was employed in the biologic systems described below.

Lethality experimental procedures

Ozone concentration-cell survival studies. Concentration-survival experiments were carried out according to the experimental protocol shown in table I. Twenty-ml. aliquots of the stock ozonated-water solutions, volumetrically pipetted, were successively delivered to each of 3 tubes (30 x 200 mm.). Tubes 1, 3, and 4 (blank containing 20 ml. distilled water) were analyzed for ozone content as described above. Tube 2, the biologic system, contained 1.0 ml. of the standardized bacterial suspension. After 5 minutes' contact time between the bacteria and the ozonated-water solution, 1.0 ml. of 1 N sodium thiosulfate was added to destroy any remaining oxidizing residue. Appropriate M/15 phosphate buffer dilutions were carried out and 0.1-ml. aliquots were delivered to quadruplicate eosin-methylene-blue (EMB) agar

plates by the spread-plate technic, to determine bacterial survival numbers after the plates had been incubated at 37° C. for approximately 24 hours. This procedure constituted a single run in an experiment. The number of runs in a given experiment varied. Each experiment, with reference to ozone-cell contact, was completed within the same day. Various concentrations of ozone in aqueous solution were achieved through manipulations of electrical input to the ozonator and total gas volume through the aqueous solution. The control run or 100 percent survival for each system was accomplished by using stock oxygenated-water solutions and carrying out identical procedures. Oxygen did not affect survival results which were similar to those obtained when air was used. Occasionally, two biologic tubes were incorporated into each run of an experiment. The two survival counts of each run were averaged and the mean bacterial count was used in calculations.

Ozone-cell, contact time-survival studies. These experiments were designed utilizing a 4 x 4 Latin square. A typical protocol is shown in table II. Twenty-ml. aliquots of the stock

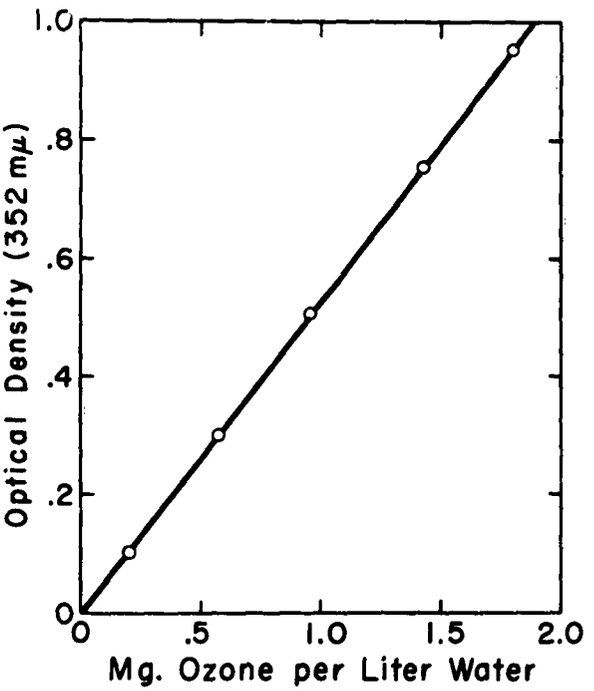

FIGURE 4

Standard spectrophotometric curve for ozone: iodine in 10 percent alkaline potassium iodide.

TABLE I

A typical experimental protocol to test the lethality of ozonated-water on cells of E. coli

Reagents and procedures	Tube number			
	1	2	3	4
	Chemical analyses	Biologic analyses§	Chemical analyses	Blank
Alkaline potassium iodide, 10% (ml.)	5	—	5	5
Water (ml.)	—	—	—	20
Cells (ml.)	—	1.0	—	—
O_2–H_2O or O_3–H_2O (ml.)*	20	20	20	—
Acetic acid, 3 N (ml.)†	1.5	—	1.5	1.5
OD (352 mμ.)				
4 min.	0.176	—	0.193	0.004
20 min.‡	0.181	—	0.198	0.009

*After 5 minutes' contact time, 1.0 ml. 1 N sodium thiosulfate added.

†Zero time — immediately after acidification.

‡Calculations for ozone concentration: Net average OD = 0.1805. Milligrams O_3/liter water = 0.345 × 1.26 dilution correction factor. Micrograms O_3/ml. biologic system = 0.435.

§Biologic control tube contained oxygenated-water.

TABLE II

A typical Latin square experimental protocol to test the effect of varying ozone-cell contact times on the survival of E. coli *in ozonated-water*

Reagents and procedures		Tube number						
		1	2	3	4	5	6	7
		Chemical analyses	Biologic analyses§	Biologic analyses	Biologic analyses	Biologic analyses	Chemical analyses	Blank
Alkaline potassium iodide, 10% (ml.)		5	—	—	—	—	5	5
Water (ml.)		—	—	—	—	—	—	20
Cells (ml.)		—	1.0	1.0	1.0	1.0	—	—
O_2–H_2O or O_3–H_2O (ml.)		20	20	20	20	20	20	—
Acetic acid, 3 N (ml.)*		1.5	—	—	—	—	1.5	1.5
Cells–O_3 contact time (min.)†	Run 1	—	1	5	10	25	—	—
	Run 2	—	5	10	25	1	—	—
	Run 3	—	10	25	1	5	—	—
	Run 4	—	25	1	5	10	—	—
OD (352 mμ.)	4 min.	0.202	—	—	—	—	0.220	0.005
	20 min.‡	0.204	—	—	—	—	0.222	0.007

*Zero time — immediately after acidification.

†After indicated cells-ozone contact time, 1.0 ml. 1 N sodium thiosulfate added.

‡Calculations for ozone concentration: Net average OD = 0.206. Milligrams O_3/liter water = 0.390 × 1.26 dilution correction factor. Micrograms O_3/ml. biologic system = 0.492.

§Biologic control tube contained oxygenated-water.

ozonated-water solution, volumetrically pipetted, were successively delivered to 6 tubes (30 x 200 mm.). Tubes, 1, 6, and 7 (the latter was the blank containing 20 ml. of distilled water) were analyzed chemically in accordance with the procedure for ozone analysis. Tubes 2 through 5 contained 1.0 ml. of the standardized bacterial suspension. Contact times between the ozonated-water and cells were 1, 5, 10, and 25 minutes. As shown in the protocol, the biologic tubes of each successive run in an experiment were rotated to provide a measurement of the physical order parameter. At the termination of a contact period, 1.0 ml. of 1 N sodium thiosulfate was added to the tube to destroy any remaining oxidizing residue. Tenfold serial dilutions with M/15 phosphate buffer were carried out and quadruplicate EMB plates of the appropriate dilutions for each time period were completed by use of the spread-plate technic. Following incubation at 37° C. for 24 hours, colonies on the plates were counted by means of a Quebec colony counter or a low-power dissecting microscope. Results were recorded as total number of viable bacteria per milliliter of the biologic system. Control experiments, establishing the 100 percent survival baseline, were done using oxygenated-water in an identical procedure as the other systems. Percent survivals were calculated. These data were analyzed for variance by established statistical methods (22).

Effect of varying cell concentration studies. The parameter of bacterial cell numbers was investigated. The procedure previously described for the ozone concentration-cell survival studies was utilized. Tenfold dilutions of the stock bacterial suspensions were employed. The range of cell concentration varied between 5×10^8 to 5×10^5 cells per milliliter of the biologic system.

Growth curve experimental procedures

Cultures of *E. coli*, strain B/r, were prepared and standardized according to procedures previously described. A typical experiment is

shown in table I. Following ozone exposure, appropriate dilutions were made and 0.1-ml. aliquots of cells from the biologic system were spread on each of several nutrient agar plates. These plates were incubated at 37° C. for a given period. After each specified period of incubation, two plates were removed from the incubator and chilled in the freezing compartment of a refrigerator for 5 minutes and then on the refrigerator shelf for an additional 10 minutes. The plates were removed from the refrigerator and the growth on each separately washed with 10 ml. of nutrient broth. Appropriate nutrient broth dilutions were made and cell assays completed on triplicate nutrient agar plates. Colony counts were made following 24 hours' incubation at 37° C. Control cultures were similarly handled.

RESULTS

Survival of cells exposed to ozonated-water as a function of initial cell concentration

The extreme variations in percent survival encountered by this investigator in early experiments on the effects of ozone concentration and contact time suggested that a portion of this variation might be due to differences in the concentration of cells. Accordingly, an experiment was designed wherein several concentrations of cells were each exposed in replicate tubes to ozonated-water. Contact time between cells and ozone was kept constant at 5 minutes. Other experimental details are described under "Materials and Methods."

When the logarithms of the initial and surviving bacterial counts were plotted as shown in figure 5, the points, fitted by eye, suggested a curve composed of two linear segments with a change in slope at approximately 7.5 (log of initial cell concentration). The slope of the lower part of the curve was approximately 2.0; the upper segment slope was estimated as 3.0. This plot held true for both strains of *E. coli*. This curve suggested that the data plotted in the two segments of the curve increased at constant ratios. If a geometric progression is formed by the ordinate values when the abscissa values are arranged geometrically, the equations may be expressed as

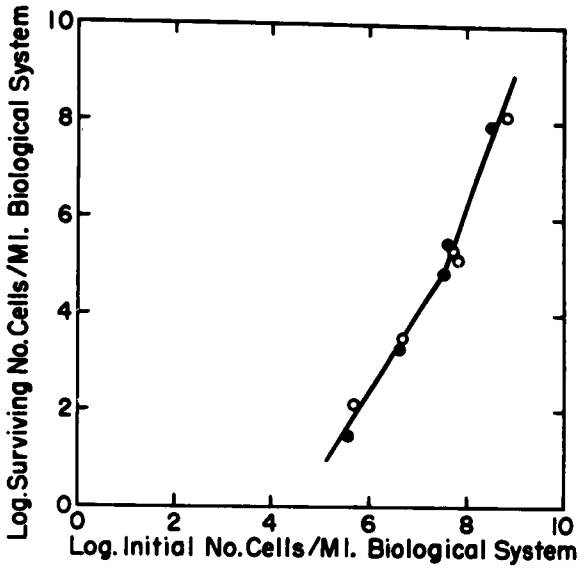

FIGURE 5

Effect of varying cell concentrations on survival of E. coli *after contact with ozonated-water. Ozone: 0.083 ± 0.008 μg. per milliliter of the biologic system. Strain B: solid circles; strain B/r: open circles.*

$$Y = a X^b \quad (1)$$

$$\log Y = \log a + b \log X \quad (2)$$

which, when plotted on logarithmic paper results in linearity. In these equations, X and Y are the number of initial and surviving cells per milliliter of the biologic system, respectively, constant a is the Y intercept when X is zero, and constant b is the slope of the fitted curve.

Since the numbers of experimental points were considered too few to gage this phenomenon, supportive data were utilized. These data were derived from experiments other than those basically designed to measure the cell concentration parameter. The logarithms of the initial and surviving number of cells per milliliter of the biologic system are plotted in figure 6. These data are distributed around two mean ozone values—namely, 0.111 ± 0.028 and 1.74 ± 0.49 μg. per milliliter of the biologic

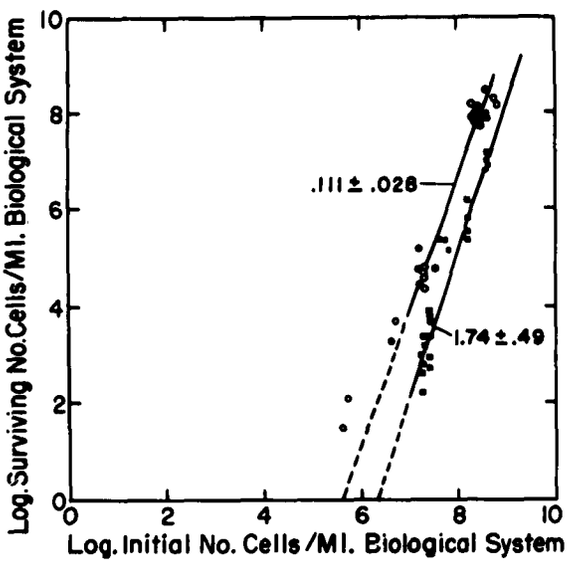

FIGURE 6

Effect of varying cell concentrations on survival of E. coli *after contact with ozonated-water. Strain B: solid circles and squares; strain B/r: open circles and squares. Ozone given in micrograms per milliliter of the biologic system, as shown. Solid curves fitted by least squares method.*

system, respectively. The curves shown have been fitted by the least squares method.

The equation for the fitted curve for the lower ozone value in figure 6 was

$$\log Y = -15.37 + 2.75 \log X \qquad (3)$$

and for the ozone value, 1.74 ± 0.49 μg. per milliliter of the biologic system,

$$\log Y = -19.69 + 3.11 \log X \qquad (4)$$

These equations permit the observation that cells exposed to ozonated-water for a 5-minute contact period increase in the number of cells surviving as a function of approximately the cube of the increasing initial cell concentration. This observation seems to hold true over a wide range of ozone values—namely, 0.111 ± 0.028 to 1.74 ± 0.49 μg. per milliliter of the biologic system. This is justified if one assumes that the two curves drawn are representatives of a family of parallel curves, and that the slopes of the fitted lines—namely, 2.75 and 3.11—are equal, within experimental error. Further, it may be stated that, under the experimental conditions employed, the initial cell volume exposed to ozone constituted a highly critical factor in determining survival results. This is based on the fact that in biologic survival studies of all types, maximum reproducibility and reliability lie within the survival range of 1 to 100 percent. The solid portions of the curves cover a narrow range of initial cell numbers per milliliter of the biologic system—namely, 1×10^7 to 1×10^9. This represents a range of survival, at least for the higher ozone value, of approximately 0.001 to 1.00 percent. The 1 to 100 percent survival range is even more limited with regard to initial cell concentration—namely, 4×10^7 to 6.3×10^8 for the lower ozone value, and 2.5×10^8 to 2.0×10^9 for the higher ozone value. The upper portions of these curves probably form a 45-degree angle with a line parallel to the abscissa as they approach 100 percent survival. The lower broken line part of each curve can only be extrapolated. It is quite probable that, as the curve approaches zero percent survival, it is asymptotic to a line parallel to the abscissa.

The results presented do not show any apparent difference in the behavior of the two strains of *E. coli* examined. Strains B and B/r appear to act similarly in their survival response as increasing concentrations of cells were exposed to a fixed amount of ozone.

Survival of cells exposed to ozonated-water as a function of ozone concentration

Early attempts by this investigator to quantitatively evaluate the lethality of ozone for *E. coli* with respect to varying ozone concentrations resulted in a series of experimental points which, when plotted, showed an excessive degree of scatter. This variation was greater than that which could be accounted for by inherent biologic variation. Since the experimental technics were considered refined to a satisfactory level to permit their use in this type of experimentation, it was concluded that some unknown factor or a combination of factors were interacting in such a manner as to cause this extreme experimental variation. One

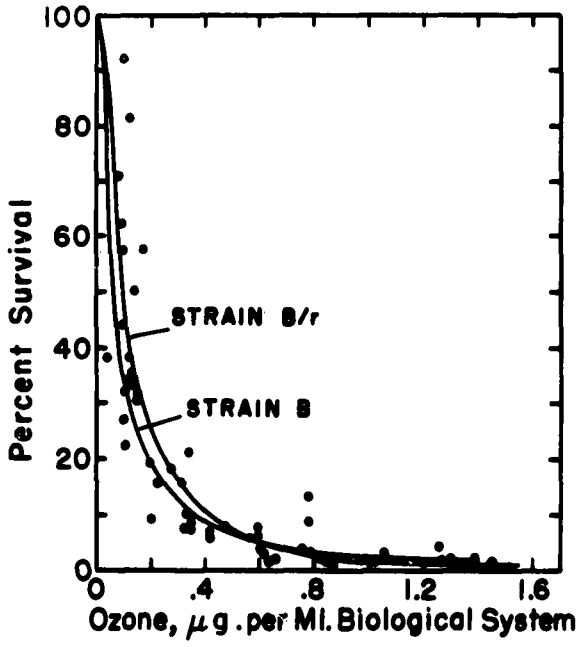

FIGURE 7

Survival of E. coli *after 5 minutes' exposure to varying concentrations of ozonated-water. Strain B: open circles; strain B/r: solid circles. Curves fitted by probit analysis. Range of initial cell concentration per milliliter of the biologic system: strain B, 2.52 × 10^8 —3.82 × 10^8; strain B/r, 0.86 × 10^8 —2.27 × 10^8.*

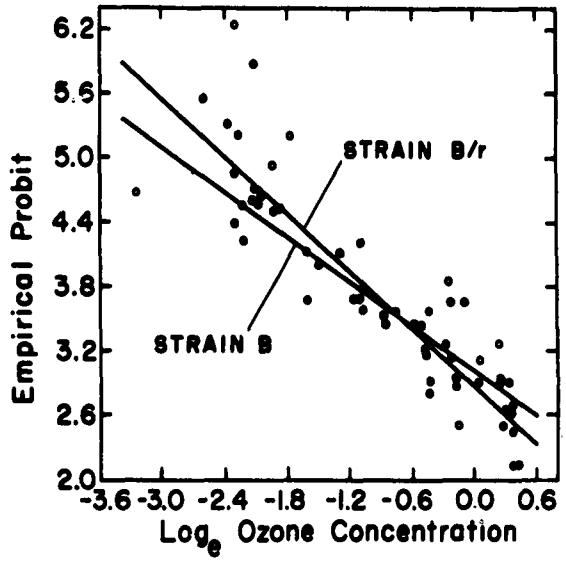

FIGURE 8

Probit plot of percent survival of E. coli *after 5 minutes of exposure to varying concentrations of ozonated-water. Ozone concentration in micrograms per milliliter of the biologic system. Strain B; open circles; strain B/r: solid circles. Curves fitted by probit analysis.*

of these factors was found to be the initial concentration of cells in the biologic system, as previously discussed. An appreciation of the critical role of initial cell concentration of the biologic system upon survival permitted a re-evaluation of some of the ozone concentration-cell survival data already accumulated. In addition, several new experiments were carried out. When these data are plotted as shown in figure 7, large variations in percent survival for essentially the same amount of ozone may be observed. Since the cell count error is probably not large, the extreme variation observed in the response is not likely that of percent survival determination. A plausible explanation may be that during the preparation of ozonated-water, an oxidant(s) is formed which interferes in ozone analytic procedure. Evidence for such a factor is presented in another paper (23).

The experimental data were submitted to a statistical evaluation. Several possible models were considered for the mode of ozone activity on the cell. Since no specific a priori notions are available about methods of the death process and, further, because of the extreme variation of the data, the only assumption made is that tolerance to log concentration of ozone is normally distributed in the population. If this assumption holds true, a probit transformation should linearize the data—that is, the straight line

$$Y = \alpha + \beta X + \epsilon \qquad (5)$$

should fit the data. In this equation Y equals the probit, X is the log concentration of ozone to the Napierian base e, and ϵ is the experimental error. These probit plots for strains B and B/r are shown in figure 8. The fitted equations for these two strains and other statistical data are summarized in table III.

The fit of the data show significant deviations from linearity at the 1 percent level.

TABLE III

Summary of probit analysis for survival data for E. coli *after 5 minutes' exposure to varying concentrations of ozonated-water*

Statistics	Strain B	Strain B/r
Fitted equation ($Y = \alpha + \beta X$)	$Y = 2.985 - 0.696X$	$Y = 2.872 - 0.897X$
Chi-square (measure of goodness of fit)	11,784.75 (31 degrees of freedom)	2,606.09 (22 degrees of freedom)
LD_{50}* (with approximately 95% fiducial limits)	0.055 (0.022 to 0.091)	0.093 (0.061 to 0.128)

*Ozone concentration, micrograms per milliliter biologic system.

This does not in itself suggest that the model is inadequate. Since the deviations about the straight line appear to be randomly distributed, the inference is that these deviations can probably be attributed to the large experimental error caused by variations in the initial cell concentrations in the several experiments and by the interference of an unknown oxidant(s) during the ozone analytic procedure, as reported in another paper (23), and that this model of normally distributed tolerance to ozone in the population is, at least, not refuted by the data.

The fitted percent survival curves shown in figure 7 are estimates of the lethal effects of ozone over a given range of initial cell concentrations—namely, $2.52 \times 10^8 - 3.82 \times 10^8$ and $0.86 \times 10^8 - 2.27 \times 10^8$, for strains B and B/r, respectively. These curves suggest that when such cell populations are exposed to ozonated-water, lethal effect increases dramatically over a narrow range of ozone values. The LD_{50} (that is, the concentrations of ozone which will kill 50 percent of approximately 1.5×10^8 cells per milliliter in 5 minutes) are 0.055 and 0.093 μg. per milliliter of the biologic system for strains B and B/r, respectively. Limited evidence is available which suggests that with a series of initial cell concentrations, each at varying concentrations of ozone, a family of curves may be shown similar to and positioned above or below those shown in figure 7, depending upon the cell concentration.

The assumption of a distribution of tolerances is a general assumption that covers several of the hit theories. In this more general formulation, one does not specify the kind of targets involved in the process nor the number of targets, if constant, or the form of the distribution if targets are not constant from cell to cell. Neither does one obtain information about the average number of targets or the fraction of total targets that must be hit before death of the cell occurs. Simply assuming a varying tolerance among the cell population allows for a variety of possible explanations about the mechanism involved in the kill process. With the extreme variation of the data, however, more specific models probably cannot be verified nor justified from the data alone. That is to say, one model will fit about as well as another but, in no instances will the fit be good. Also, one of several models will not be distinguishable from the others, judging from the fit of the data itself.

Finally, it is noted from figure 7 that strains B and B/r of *E. coli*, when exposed to varying concentrations of ozone, appear to behave in a similar, if not an identical, manner.

Survival of cells exposed to ozonated-water as a function of ozone-cell contact time

The time parameter as a factor in cell survival following exposure to ozonated-water was examined. A series of experiments was

designed utilizing a 4 x 4 Latin square. Three controlled or predicted variables were included in this design—namely, time, order, and ozone concentration. The times for ozone-cell contact periods were arbitrarily set at 1, 5, 10, and 25 minutes. The order variable refers to the physical, numerical position of any biologic tube during any given experimental run. An estimate of the variability of order was necessary after it was found that aliquots of ozonated-water, delivered successively to several tubes, decreased successively in ozone concentration even though concentration differences between tubes were of apparently minimal order. The effect of the concentration of ozone on percent survival for each replicated run during an experiment was also tested by variance analysis procedures. The technical details have been described fully under "Materials and Methods."

A total of three experiments comprising twelve runs was carried out for each of the two strains of *E. coli*, B and B/r. Table IV is representative of the manner in which the basic data (percent survivals) were handled. Percent survivals were carried out to the fourth decimal place in order to do the analysis of variance. An analysis of variance was completed for each experiment. Table V shows a typical example. Probability of significance in the analyses was set at the 5 percent level.

TABLE IV

Percent survival of E. coli, *strain B, following exposure to ozonated-water for varying time periods (arranged in a Latin square)*

Time (min.)	Ozone, μg./ml. biologic system				Mean percent survival
	1.05	1.30	1.39	1.25	
1	A: 1.1813*	B: 4.3956	C: 1.3736	D: 1.0440	1.9986
5	B: 0.8791	C: 1.9231	D: 1.8407	A: 4.1209	2.1910
10	C: 0.6319	D: 2.6923	A: 2.1429	B: 3.8736	2.3352
25	D: 0.6319	A: 1.8956	B: 0.7418	C: 1.8681	1.2844
Mean percent survival	0.8311	2.7267	1.5248	2.7267	—

Summary of physical order of biologic tubes

Mean percent survival	A	B	C	D	—
	1.7789	1.3393	2.0055	2.6855	—

Physical order of biologic tubes in each run: A = 1 minute, B = 5 minutes, C = 10 minutes, D = 25 minutes.

*100 percent survival = 3.64×10^6 cells/ml. biologic system.

TABLE V

Analysis of variance of percent survival of E. coli, *strain B, after exposure to ozonated-water for varying time periods*

Source of variance	Degrees of freedom	Mean square	F	P
Time	3	0.86907	0.884	NS*
Order	3	1.26164	1.287	NS
Ozone	3	3.51900	3.590	NS
Error	6	0.98031	—	—

*NS — Not significant at the 5 percent level.

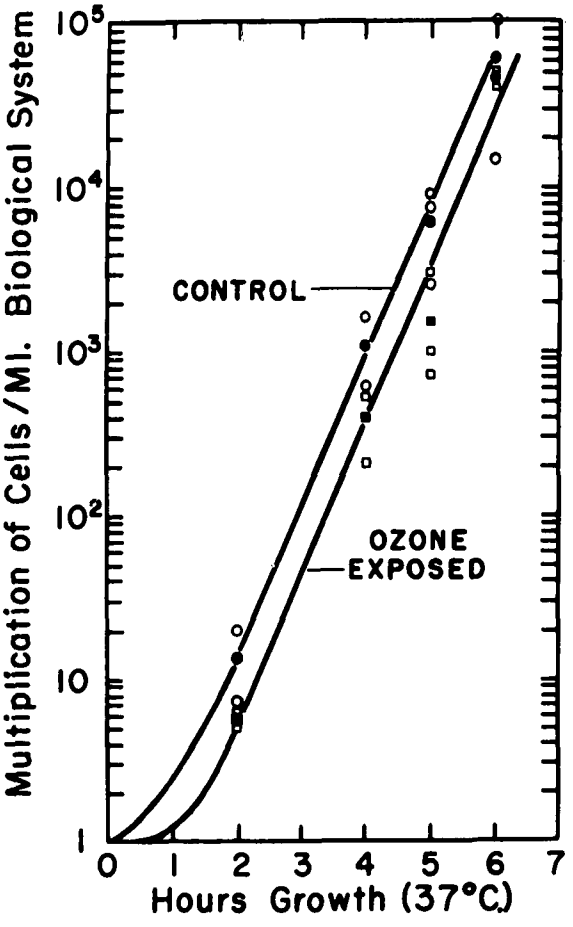

FIGURE 9

Growth rate of E. coli, *strain B/r, on nutrient agar after exposure to ozonated-water. Control cells: open circles; ozone-exposed cells: open squares. Means: solid circles and squares.*

In these experiments, times of ozone exposure of the bacterial population greater than 1 minute had no significant effect on lethality processes as measured by the numbers of surviving cells. Contact periods between the cells and the ozonated-water ranging between 1 and 25 minutes showed no significant difference in the number of cells killed at each of the three ozone concentrations employed. The concentrations of ozone effecting lethality between 63 and 99.5 percent ranged between 0.1 to 1.5 μg. per milliliter of the biologic system. These results are equally applicable to both strains of *E. coli*, B and B/r.

In general, the physical arrangement of the biologic tubes during the experimental procedure did not interpose significant variance into the experimental design. This order variance was significant in only one of the six experiments ($P<.025$).

Previous experiments already have shown the inherent technical difficulties in producing replications of given concentrations of ozone in water under the experimental conditions employed in these studies. Percent survivals during an experiment varied significantly among the four runs in each experiment in two of the six experiments carried out.

A comparison of the two strains of *E. coli*, B and B/r, for survival differences is difficult with the data presented here because of the variations in ozone concentrations among the several runs in appropriately compared experiments. The t-test, however, was applied to the survival data from two reasonably comparable experiments. In these experiments, strains B and B/r were exposed to mean ozone concentrations of 0.123 and 0.106 μg. per milliliter, respectively, of the biologic system. The t-value for the strain comparison was 1.9141. This was not significant at the 5 percent level ($t_{0.05} = 2.056$, 26 degrees of freedom). These results suggest that strains B and B/r do not differ in their tolerance to ozone, at least at the level of ozone applied.

Growth characteristics of cells surviving contact with ozonated-water

A comparison of the growth rates of control and ozone-exposed cells is shown in figure 9. The parallel portions of the curves suggest that the growth rate of ozone-exposed cells is the same as that of the control cells. The only difference appears to be in the first hour or so of incubation. This difference can be explained by a longer lag phase for the ozone-exposed population. This longer lag phase is due to the lower concentration of initially viable cells (24).

Further evidence of the different lag phases of the ozone-exposed and control cells may be

seen in the growth curves in figure 10. Time lag may be calculated from the curves. A line is drawn parallel to the slope of each of the logarithmic phases of the curves to intersect the ordinate at a point representing the size of the inoculum at zero time. The horizontal distance between this line and the growth curve represents the duration of the lag phase. The time lags for the ozone-exposed and control cells were calculated at 75 and 36 minutes, respectively. It is pointed out again that this increased lag phase exhibited by the ozone-exposed cells is probably due to the initially lower number of viable cells. Using the equations described by Gunsalus (24), generation times were calculated for both the ozone-exposed and control cells. These generation times were 19 minutes for both types of cells.

It is reasonable to state that under the experimental conditions employed, there were no observable differences in the growth characteristics of the ozone-exposed cells and the control cells.

DISCUSSION

Some factors which contribute to an understanding of the biologic activity of ozone

The *modus operandi* of the ozone molecule conforms to rigid universal laws. In order to understand its biologic mode of action, it is necessary to approach an appreciation of the characteristics of this molecule on the less complex, nonbiologic level. Since universal laws hold true, knowledge accumulated at the nonbiologic level may be applied to the biologic entity.

One may consider the ozone molecule in relation to a few of the many contributing factors that fashion its biologic activity, as shown in figure 11. Spontaneous decomposition of ozone in aqueous solution has been extensively studied with divergent results and concomitant theories to explain them (25-33). Studies by this investigator (34) appear to indicate a first order reaction for the spontaneous decomposition of ozone in aqueous solutions. Furthermore, the rate of decomposition is decreased with increasing acidity. The

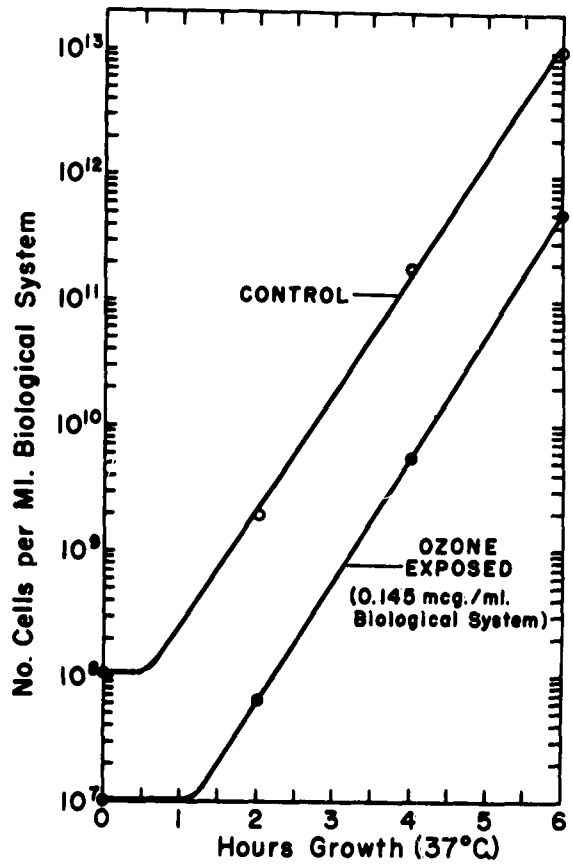

FIGURE 10

Growth curve of E. coli, *strain B/r, after exposure to ozonated-water. Control cells: open circles; ozone-exposed cells: solid circles.*

biologic application of these data would suggest that ozone lethality should increase with decreasing acidity. This has, indeed, been reported (35). Considerations which would influence the rate of spontaneous decomposition of ozone and, as such, its biologic activity, are time, temperature, concentration, and others.

Another factor that requires some knowledge, because of the role it plays in biologic experimentation, is the physicochemical activity of ozone on nonviable or inert surfaces. To explain this, let us consider what occurs when an aliquot of ozonated-water is transferred from a primary vessel to another by means of a pipet. Assuming that all glassware

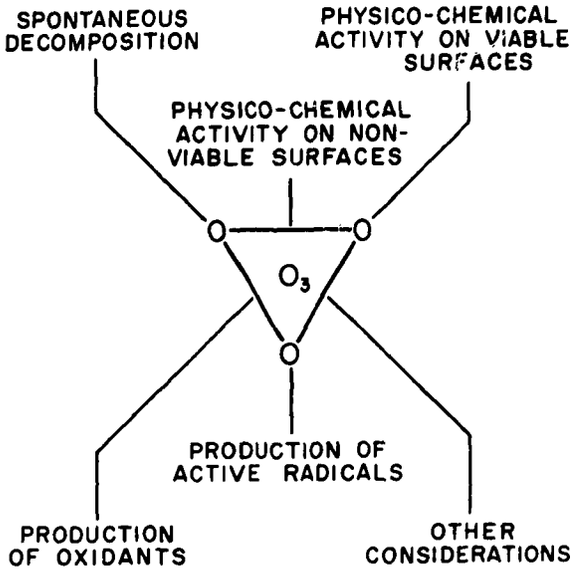

FIGURE 11

Some factors which contribute to an understanding of the biologic activity of ozone.

is scrupulously clean, if one determines the ozone concentration in each of the two vessels, it is soon apparent that the amount in the aliquot is considerably less than that theoretically pipetted from the primary vessel. The explanation resides in the fact that ozone molecules are physically destroyed each time they contact a surface. In this case the glass surfaces represent a chemically inert substance. Another example in which chemical and physical forces may be envisioned is the significant decrease in ozone concentration when an aliquot of ozonated-water is diluted into an equal aliquot of water. Such experiments are recorded in table VI.

TABLE VI

Effect of dilution on the ozone concentration of ozonated-water solutions

Ozone, milligrams per liter of water			Actual percent ozone remaining of theoretic concentration
Initial	After dilution		
	Theoretic	Actual	
1.900	0.950	0.218	23
1.650	0.825	0.195	23
1.161	0.581	0.076	13

The formation of active radicals during the decomposition of ozone in water has been studied by several investigators (26, 27, 28, 36). Suffice it to state that the hydroxyl radical appears to be the most reasonable intermediate in such reactions (28) and it has been proposed as an intermediate in many reactions in aqueous solution (37). The production of oxidants, especially hydrogen peroxide, has been theoretically considered (33). Proof of such occurrence, however, is lacking in the literature. Experimental evidence (23) indicates that when ozone is absorbed in aqueous solution, oxidants are formed which do not seem to be identifiable as hydrogen peroxide.

Returning to the consideration of the factors shown in figure 11, it is clear that information on the nonbiologic production of active radicals and oxidants permits interpretations and studies on the biologic level. Evidence of such research is well documented (38, 39). Finally, some knowledge of the numerous other factors which contribute to the biologic effect of ozone permits an evaluation of the experimental results set forth in this paper.

SUMMARY

Evidence for the functioning of certain critical factors during the killing process is presented. Maximum lethal effects of ozone over a wide concentration range were expressed in 1 minute or less on the bacterial population. Tolerance to the log concentration of ozone appears to be normally distributed in the population. The number of cells surviving ozone exposure is a function of approximately the cube of the initial cell concentration. Finally, the ozone tolerance of strains B and B/r of *E. coli* appears to be similar, if not identical.

The growth characteristics of cells surviving the lethal effects of ozone were studied. There were no observable differences between the cells exposed to ozone and the control cells.

Several factors which contribute to an understanding of the biologic activity of ozone were analyzed. The importance of free radicals in biologic processes was discussed. A review of the literature on the formation of these radicals during the absorption of ozone in aqueous solution is presented.

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